J. HYM. RES.
Vol. 17(2), 2008, pp. 195–200

Evaluation of Specimen Preservatives for DNA Analyses of Bees

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Abstract.—Large-scale insect collecting efforts that are facilitated by the use of pan traps result in large numbers of specimens being collected. Storage of these specimens can be problematic if space and equipment are limited. In this study, we investigated the effects of various preservatives (alcohol solutions and DMSO) on the amount and quality of DNA extracted from bees (specifically Halictidae, Apidae, and Andrenidae). In addition, we examined the amount and quality of DNA obtained from bee specimens killed and stored at −80°C and from specimens stored for up to 24 years in ethanol. DNA quality was measured in terms of how well it could be PCR-amplified using a set of mitochondrial primers that are commonly used in insect molecular systematics. Overall the best methods of preservation were ultra-cold freezing and dimethyl sulfoxide, but these are both expensive and in the case of ultra-cold freezing, somewhat impractical for field entomologists. Additionally, dimethyl sulfoxide was shown to have adverse effects on morphological characters that are typically used for identification to the level of species. We therefore recommend that the best alternative is 95% ethanol, as it preserves bee specimens well for both morphological and molecular studies.

Recent advances in insect molecular systematics, made possible by the polymerase chain reaction (PCR) and other molecular techniques have made it important to properly preserve rare specimens for maintaining museum collections, or for molecular analysis. Since DNA can be damaged by enzymatic breakdown, oxidation and hydrolysis (Lindahl 1993, Quicke et al. 1999), specimens need to be preserved from the time of collection to the time of analysis in order to minimize DNA degradation. Several factors have been reported that affect DNA degradation in stored insect specimens, including preservative type and concentration, time in preservative, temperature, pH, and the age of the specimen (Dillon et al. 1996). It is generally accepted that the highest quality of DNA is extracted from live specimens (Tayutivutikul et al. 2003), live specimens frozen at −80°C (Dillon et al. 1996), or live specimens quick frozen in liquid nitrogen (Quicke et al. 1999). However, these methods are not always practical for field biologists, and several alternatives have been reported for preserving arthropod, mammalian or plant specimens for the purpose of genetic analysis. These include storage in preservatives such as methanol, ethanol, and isopropanol (Post et al. 1993), propylene glycol (Rubink et al. 2003), acetone (Fukatsu 1999), Carnoy's solution (Post et al. 1993), and dimethyl sulfoxide (Kilpatrick 2002). An important motivation for the current study is an increasing research emphasis on large-scale collections of bees, especially using pan traps that generate specimens used for molecular systematics and population genetic studies. We therefore needed to assess the relative merits of various preservatives since specimens may have to be preserved for considerable periods of time before being analysed.

While few studies of the efficacy of the various preservation methods have fo-

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cused on collections of bees, other insect groups have been well represented, but many of these studies focused only on DNA preservation, or morphology. King and Porter (2004) determined that 95% ethanol or 95% isopropanol were ideal for preserving ants for card-point mounting and through a literature review concluded that DNA was best preserved in 95% ethanol. Quicke et al. (1998), studying wasps of the superfamilies Ichneumonoidea and Chalcidoidea, had an 80% success rate at PCR amplification of 28S rDNA from specimens preserved in 70% ethanol at room temperature. Austin and Dillon (1997) also suggested that chemical drying methods could be used to generate sufficient quantities of quality DNA from ichneumonoid and chalcidoid wasps. In this report we present our findings on the relative efficacies of several storage methods of bees including ultra cold freezing, various alcohols, and pure dimethyl sulfoxide (DMSO) in terms of the quality of DNA preserved for downstream applications such as PCR, as well as the ability to preserve delicate morphological features that are required for taxonomic identification. We analyzed 121 individuals representing three families, Halictidae, Apidae, and Andrenidae, as part of several larger studies of bee diversity. To determine the quality of the DNA that was extracted using each of our preservation methods, three amplicons within the mitochondrially encoded gene, *cytochrome oxidase subunit I* (COI), a gene widely used in insect systematics, were compared. While the majority of the specimens collected for this study were part of a pre-planned experiment, we supplemented the data using older, preserved specimens that had been previously collected in the course of other projects.

MATERIALS AND METHODS

Most of the bee specimens used in this study were collected in southern Ontario, Canada and Maryland, USA, using pan traps containing a mixture of water and blue Dawn® dish detergent. Pan traps were placed approximately 10 m apart along transects and were subject to both sun and shade conditions for between 6 and 24 hours. Bees were identified and then transferred to methanol (50 or 95% in water), ethanol (50, 70 or 95% in water), an ethanol-methanol solution (70:30, 95:5), or pure DMSO. Bees were stored at room temperature for between one and twelve months after which they were pinned and stored at room temperature until DNA was extracted.

In addition to these specimens, we also analyzed specimens that were live caught in nets. These included eight *Xylocopa virginica* that were killed by freezing at −80°C, four *Lasioglossum marginatum* caught in Greece and stored in ethanol since 1994, and 24 *Halictus poeyi* caught in Florida and stored in 70% ethanol since 1982. These latter bees, as well as those stored in DMSO, remained in preservative, or frozen, until DNA was extracted. Prior to DNA extraction, bees were dried overnight at room temperature to remove any remaining preservative. Bees preserved in DMSO were washed with 95% ethanol, and then allowed to dry overnight at room temperature.

DNA was extracted using either the Qiagen DNeasy Tissue Kit (insect protocol) or the Sigma-Aldrich GenElute Mammalian Genomic DNA Purification Kit, following the manufacturers' instructions. The quality of the extracted DNA was assessed by agarose gel electrophoresis of 5 uL of sample on 1.2% agarose gels in TAE buffer containing 10 mg/ml ethidium bromide. Total DNA extracted from each specimen was quantified using a Beckman DU-530 spectrophotometer. DNA extractions were based on whole specimens, with the exception of *X. virginica*, a very large bee species, for which a single leg was used. The effect of body size on the amount of DNA extracted from bees of different species was statistically controlled by

measuring the average head width of a set of pinned specimens (in halictid and xylocopine bees, head width is highly correlated with body mass).

Polymerase chain reaction (PCR) was carried out to determine the quality of the preserved DNA. COI amplicons were amplified using the primers Ron (C1-J-1751), Nancy (C1-N-2191), Jerry (C1-J-2183), and Pat (L2-N-3014) (Simon et al. 1994). The first PCR reaction was performed with the Ron-Pat primer pair generating an amplicon of 1264 bp. Upon successful amplification of the 1264 bp amplicon, specimens were removed from any further attempts at amplification. For those specimens that did not successfully amplify using the Ron-Pat primers, a second PCR reaction was performed on 1:100 dilutions of the Ron-Pat PCR product and used either the Ron-Nancy or Jerry-Pat primers.

The quality and quantity of PCR amplification products were analyzed by agarose gel electrophoresis as described above. Successful amplifications were scored using a system based on the brightness of the bands present on the gel. A score of '0' was given for no amplification, a score of '1' was given for weak amplification not containing enough DNA to be sequenced, and a score of '2' was given for a bright band that would contain a sufficient quantity of DNA for sequencing (note that our lab has successfully used this scoring system for DNA sequencing for more than 5 years). Images were photographed on the Bio-Rad Gel Doc 1000 using Multi-Analyst (Bio-Rad) software.

RESULTS

Qualitative Observations

Specimens that were preserved in alcohol-based solutions were dehydrated, brittle, and easily damaged when handled. Bees preserved in DMSO were less brittle than those stored in alcohol, but many of the morphological characters that are typically used for identification were distorted; for instance, wings were shrivelled (Fig. 1). Several halictids that were stored in DMSO, notably the augochlorine bees, changed colour from bright green to reddish gold, although their natural green colour was restored after washing them in ethanol. These morphological changes proved problematic for those specimens that had not been identified prior to preservation.

DNA Quality

All preservation methods tested produced DNA of varying quality and concentration (Fig. 2). Pure DMSO was most successful at maintaining genomic quality (the brightest genomic band is Lane 8 in Fig. 2), whereas ethanol and methanol-preserved bees produced weak genomic DNA bands. All three methanol treatments, as well as the 50% ethanol and ethanol-methanol blends, showed signs of DNA degradation indicated by extensive smearing.

Several factors could affect both the amount and quality of the DNA from our specimens including species, collection method, preservative, and specimen size (Table 1). An ANCOVA analysis showed that specimen size was the strongest contributor to the DNA quantity ($F=30.03$, $df=1$, $p<0.0001$) and preservative type had only a slight effect ($F=2.10$, $df=7$, $p=0.0568$). Other potential influences (type of DNA extraction kit and time in preservative) had non-significant effects on the amount and quality of DNA obtained from specimens.

For systematics projects, the amount of DNA obtained from specimens is less important than how well the DNA can be PCR-amplified. DNA concentration and preservative type had strong effects on PCR amplification scores; the highest amplification scores were for specimens that were caught live and immediately frozen in a $-80°C$ freezer, despite the low amount of total DNA that was recovered. The best

Fig. 1. Pictures of *Augochlorella striata* that were preserved in 100% DMSO (left column) and 100% ethanol (right column). The top row indicates the colour changes that are apparent in the DMSO preserved bees. The bottom row shows how DMSO dehydrates the wings causing them to become misshapen.

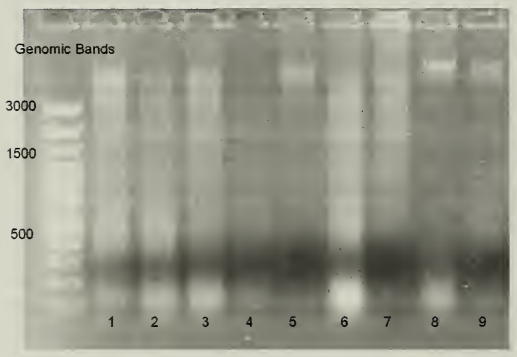

Fig. 2. Representative samples of extracted DNA from various preserved specimens separated on 1.2% agarose stained with ethidium bromide. Lanes contain the following: (1) 95% methanol, (2) 50% methanol, (3) 50% ethanol, (4) 70% ethanol, (5) 95% ethanol, (6) 70:30 ethanol-methanol, (7) 95:5 ethanol-methanol, (8) 100% DMSO, and (9) −80°C. 2.5 µg of a 100 bp size marker (Fermentas) is loaded in each of the outside lanes.

amplification scores from a liquid preservative came from those bees preserved in DMSO, followed by those preserved in 95% ethanol (lower DNA concentrations produced higher amplification scores).

As a final note, we also attempted to extract and amplify DNA from 28 specimens that had been stored in ethanol (probably 70% ethanol) for 10–24 years. These comprised four *Lasioglossum marginatum* specimens that were collected in Greece in 1994, as well as 24 *Halictus poeyi* that were collected in Florida in 1982. For three of the four *L. marginatum*, we were able to amplify only the smallest of the COI amplicons (using primers Ron and Nancy), with amplification scores of 1. Attempts at recovering DNA from the much older (and

Table 1. Mean DNA concentration, proportion of specimens scoring '2', and amplification score ranks for each of the preservative types tested.

Collection method	Preservative	No. specimens	Average DNA concentration (μg/mL) (SD)	No. specimens with amplification scores of 2
Pan traps	50% Methanol	10	138.9 (141.1)	0
	95% Ethanol	10	69.4 (26.6)	1
	50% Ethanol	10	93.8 (52.7)	3
	70% Ethanol	23	50.8 (18.9)	10
	95% Ethanol	10	56.3 (25.0)	8
	Ethanol:methanol blend 70:30	8	104.1 (60.5)	3
	Ethanol:methanol blend 95:5	10	89.6 (59.0)	3
	DMSO	8	104.3 (44.3)	6
Live caught	−80°C	8	17.0 (10.9)	8

more poorly preserved) *H. poeyi* specimens were completely unsuccessful.

DISCUSSION

The ideal preservative for field collections of bees and other insect specimens should be easy to use, cost efficient, and easily transportable. Typically, primary alcohols have been used to meet these requirements, but recent studies have examined the use of propylene glycol (Rubink et al. 2003, Vink et al. 2005), acetone (Fukatsu 1999), and other commercial products (Vink et al. 2005). Preserving the quality of both genomic and mitochondrial DNA is of great importance for conducting molecular studies. We found that ultra cold freezing was the best method for killing and preserving specimens, but this is often impractical either because specimens cannot be captured alive or because ultra cold freezing facilities are not available. The use of ultra cold freezing has been suggested by Reiss et al. (1995) and Dillon et al. (1996). In the later study, ultra cold storage of parasitic wasps did not affect the amount of DNA that could be recovered or successfully amplified.

Among the liquid preservatives DMSO serves as an ideal candidate for denaturing DNA damaging enzymes and for preserving the quality of genomic and mitochondrial DNA. The drawback to using DMSO as a preservative is that it distorts morphological characters required for identification of specimens although this problem can be overcome by identifying specimens prior to storage in DMSO. Furthermore, DMSO is considerably more expensive than ethanol. Despite the finding that primary alcohols caused advanced signs of genomic degradation, the best alternative appears to be 95% ethanol. It is relatively inexpensive, easy to transport, and does not distort morphological characters to the same degree as DMSO.

Preservative type and concentration, as well as storage time, affect the quality as well as the quantity of DNA that can be extracted from a given specimen. Additionally, some methods of preservation have adverse effects on morphological characters that need to be preserved for specimen identification. In this paper we propose that 95% ethanol is the best chemical preservative for maximizing the quantity and quality of DNA, as well as for maintaining morphological integrity when ultra-cold freezing is not immediately available.

ACKNOWLEDGEMENTS

Special thanks to T. Chessar and H. Ginsberg for reviewing the early stages of this manuscript, and S. Rehan for bee measurements. Funding was provided by Brock University, NSERC, USGS.

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